APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1991, p. 981–986
0099-2240/91/040981-06$02.00/0

Vol. 57, No. 4

The Solubility of Inclusion Proteins from *Bacillus thuringiensis* Is Dependent upon Protoxin Composition and Is a Factor in Toxicity to Insects

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Received 13 August 1990/Accepted 28 January 1991

***Bacillus thuringiensis* subsp. *aizawai* HD133 is one of several strains particularly effective against *Plodia interpunctella* selected for resistance to *B. thuringiensis* subsp. *kurstaki* HD1 (Dipel). *B. thuringiensis* subsp. *aizawai* HD133 produces inclusions containing three protoxins, CryIA(b), CryIC, and CryID, and the CryIC protoxin has been shown to be active on resistant *P. interpunctella* as well as on *Spodoptera* larvae. The CryIA(b) protoxin is very similar to the major one in *B. thuringiensis* subsp. *kurstaki* HD1, and as expected, this protoxin was inactive on resistant *P. interpunctella*. A derivative of *B. thuringiensis* subsp. *aizawai* HD133 which had been cured of a 68-kb plasmid containing the *cryIA(b)* gene produced inclusions comprising only the CryIC and CryID protoxins. Surprisingly, these inclusions were much less toxic for resistant *P. interpunctella* and two other Lepidoptera than those produced by the parental strain, whereas the soluble protoxins from these strains were equally effective. In contrast, inclusions from the two strains were about as active as soluble protoxins for *Spodoptera frugiperda* larvae, so toxicity differences between inclusions may be due to the solubilizing conditions within particular larval guts. Consistent with this hypothesis, it was found that a higher pH was required to solubilize protoxins from inclusions from the plasmid-cured strain than from *B. thuringiensis* subsp. *aizawai* HD133, a difference which is probably attributable to the absence of the CryIA(b) protoxin in the former. The interactions of structurally related protoxins within an inclusion are probably important for solubility and are thus another factor in the effectiveness of *B. thuringiensis* isolates for particular insect larvae.**

Most strains of *Bacillus thuringiensis* contain several protoxin genes encoded on large plasmids (3, 15, 20). The relative extent of synthesis of the various protoxins produced by a particular strain differs (4, 24) and is a factor in specificity. Among the Lepidopteran-active class, the CryI protoxins of 130 to 135 kDa are highly conserved in the carboxyl half and probably interact through disulfide bonds (5, 7, 17) to form the single bipyramidal inclusion found in many isolates. These inclusions are solubilized in the larval gut and are cleaved into toxins which bind to specific receptors on the cells lining the gut (13). The effectiveness of a particular toxin, therefore, is dependent on inclusion solubility, proteolytic cleavage into toxins, and affinity for available receptors. The importance of solubility (18) and receptor binding (13, 33) has been documented.

In the course of a study attempting to define the effective protoxins produced by certain strains of *B. thuringiensis* subsp. *aizawai* active on *Plodia interpunctella* selected for resistance to Dipel (25, 26), plasmid-cured derivatives of *B. thuringiensis* subsp. *aizawai* HD133 were isolated (12). It was later found that the CryIC protoxin produced by *B. thuringiensis* subsp. *aizawai* strains was responsible for the activity on resistant *P. interpunctella* (33). Nevertheless, inclusions from a plasmid-cured strain which still produced this protoxin were found to be less toxic than those from the parental strain. The basis for this lower toxicity is apparently changes in inclusion solubility. The interaction of multiple protoxins within an inclusion appears to be an additional factor in the overall effectiveness of a particular isolate.

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MATERIALS AND METHODS

Bacterial strains and growth. The properties of the strains and derivatives used are summarized in Table 1. *B. thuringiensis* subsp. *aizawai* HD133 was obtained from H. Dulmage (collection now at U.S. Department of Agriculture Northern Regional Laboratory, Peoria, Ill.). Plasmid-cured derivatives were isolated essentially as described by Jarrett (19) by growing cells for 16 h at 42°C in G-Tris medium and then streaking them on G-Tris (1) plates which were incubated at 42°C. Sporulated colonies were screened at random in the phase microscope for what appeared to be smaller inclusions, and the plasmid profiles of these presumptive cured strains were analyzed by gel electrophoresis (8). Several isolates (frequency of about 1 in 50) lacking the 68-kb plasmid were found, and one strain, designated strain 11, has been studied further. A *Bacillus cereus* transcipient (strain 9) containing the 68-kb plasmid from *B. thuringiensis* subsp. *kurstaki* HD133 was obtained by mating with *B. cereus* 569 Sm^r as previously described (2, 12).

Cloned protoxin genes and probes. Clone N_7 is a 3.4-kb *Nde*I fragment of the *cryIA(c)* gene (4). A 1.8-kb *Eco*RI-*Hin*dIII fragment from N_7 was used as a probe for the coding regions of the amino halves, and a ca. 0.8-kb *Hin*dIII-*Nde*I fragment was used for the carboxyl halves of protoxin genes. The *cryIC* gene was cloned as a ca. 7-kb *Eco*RI fragment from a digest of a crude plasmid preparation (6) from strain 11 in the *Escherichia coli-Bacillus subtilis* shuttle vector pHP13 (11) as well as in pUC18. Clones were identified

TABLE 1. Properties of strains employed

Organism	Protoxin gene composition	Source
B. thuringiensis subsp. *aizawai* HD133	CryIA(b), CryIC, CryID (CryIA cryptic)	H. Dulmage
B. thuringiensis subsp. *aizawai* 11	CryIC, CryID (CryIA cryptic)	Curing of 68-kb plasmid from *B. thuringiensis* subsp. *aizawai* HD133; see Materials and Methods
B. thuringiensis subsp. *kurstaki* HD1	CryIA(a), CryIA(b), CryIA(c), CryIIA, CryIIB	H. Dulmage (4, 24)
B. thuringiensis CG3	CryIA(a), CryIA(c), CryIIA, CryIIB	Loss of 67-kb plasmid from *B. thuringiensis* subsp. *kurstaki* HD1 (8a)
B. thuringiensis 9	CryIA(b)	Transfer of 68-kb plasmid from *B. thuringiensis* subsp. *aizawai* HD133 by mating with *B. cereus* 596 Sm^r (2, 12)
B. thuringiensis subsp. *kurstaki* HD1-9	CryIA(b)	B. Carlton; plasmid-cured derivative of *B. thuringiensis* subsp. *kurstaki* HD1 (4, 27)

initially with the N_7 probe and subsequently with a *cryIC*-specific oligonucleotide (200A; 5′ TGTTAATACTATAAC TCGTGC). Restriction enzyme digest patterns for several enzymes were identical to those of *cryIC* genes from *B. thuringiensis* subspp. *entomocidus* (16) and *aizawai* (30).

A *cryIA(b)* gene was cloned from a *Bam*HI digest of a plasmid preparation from *B. thuringiensis* subsp. *aizawai* HD133 in the shuttle vector pLP1201 (29). The 1.8-kb *Eco*RI-*Hin*dIII fragment mentioned above was used as a probe. A ca. 5-kb insert was partially sequenced as double stranded DNA (primers and Sequenase were used) in the carboxyl half to demonstrate the presence of a 78-bp deletion found in most *cryIA(b)* genes (9, 15) and through the variable region encoding amino acids 438 to 587. The 450-bp sequence of the latter was identical to that of the *cryIA(b)* genes from *B. thuringiensis* subsp. *aizawai* ICI (10) and IPL 7 (28). Several restriction enzyme sites found in all *cryIA(b)* genes sequenced to date (15) were also present in this fragment, and the clone (p121) produced a ca. 130-kDa antigen reacting with anti-CryIA(c) rabbit antibody after electroblotting in 10% urea–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (32; unpublished results). A 584-bp *Eco*RI fragment from the variable region of this cloned gene (10, 28) was used as a probe for *cryIA(b)* mRNA [with some cross hybridization to *cryIA(c)* mRNA]. A synthetic oligonucleotide designated 356 (5′ AGTAATCATTCTTTATTTGCACACC), which is a specific for the 3′ ends of *cryIA(b)* and *cryIC* mRNAs, was also used.

Inclusion purification and solubilization. Spores plus inclusions were harvested from G-Tris plates (1) after incubation at 30°C for 40 h or for 4 days at 27°C by scraping the spores from the surface of chilled petri dishes into 1 M KCl–5 mM EDTA. The pellets were washed once with deionized water containing 5 mM phenylmethylsulfonyl fluoride and twice with water (10 ml each). The pellets were finally suspended in water plus 0.2% Triton X-100 and 1% Renografin (66% meglumine diatrizoate and 10% sodium diatrizoate; Squibb). Portions were layered over step gradients consisting of 6 ml of 50% Renografin plus 4 ml of 40% Renografin in water and centrifuged in a Sorvall HB4 rotor at 8,000 rpm for 50 min. The inclusion band was removed and, if still contaminated with spores and debris, repurified through a second step gradient. The final band was diluted at least fivefold with water, pelleted at 8,000 rpm for 20 min in a Sorvall SS-1 rotor, and washed twice with deionized water before being dried in a Speed-Vac (Savant).

For solubilization, portions were weighed into Eppendorf tubes and suspended sequentially in buffers as indicated in Table 4. The initial buffer, 0.03 M Na_2CO_3–0.1% β-mercaptoethanol (βME) (pH 9.2), was selected on the basis of a preliminary screening of the conditions required to solubilize most of the protein from inclusions from *B. thuringiensis* subsp. *aizawai* HD133 or *B. thuringiensis* subsp. *kurstaki* HD1. More reducing conditions at a higher pH were then used, and finally 6 M urea–1% SDS–0.05 M dithioerythritol–2 mM phenylmethylsulfonyl fluoride (pH 9.6) (UDS-PMSF), which solubilizes essentially all of the inclusion protein, was used. The initial inclusion suspensions were heated at 65°C for 2 min to inactivate residual proteases, a treatment which did not lower toxicity. Incubations were at 37°C for 20 min, and the extractions were repeated once or twice. The supernatants from each extraction were pooled, portions were precipitated in 10% trichloracetic acid, and the pellets were dissolved in 0.2 N NaOH for protein assays (BCA reagent; Pierce Chemical Co.). Another sample was taken for fractionation in 10% urea–SDS-PAGE with silver staining (kit from Accurate Chemical Corp., San Diego, Calif.).

Bioassays. For bioassays of *Trichoplusia ni*, *Manduca sexta*, *Heliothis virescens*, and *Spodoptera frugiperda*, protoxins solubilized in 0.03 M Na_2CO_3–2% βME (pH 9.7) were

TABLE 2. Relative toxicity of inclusions and soluble protoxins

Source of inclusions or soluble protoxins	LD_{50}[a] for:			
	M. sexta	*T. ni*	*S. frugiperda*	*H. virescens*
HD133 inclusions	0.12	0.35	1.7	0.15
HD133 soluble	0.10	0.25	1.6	0.15
Strain 11 inclusions	0.65	1.00	1.3	>10
Strain 11 soluble	0.12	0.16	0.6	>10
B. thuringiensis subsp. *kurstaki* HD1 inclusions	<0.05	0.07		0.06
B. thuringiensis subsp. *kurstaki* HD1 soluble	<0.06	0.08		
Strain CG3 inclusions		0.55		
Strain CG3 soluble		0.24		

[a] Values reported are per microgram of 130- to 135-kDa protein (averages of two experiments with ranges of ±30%). The total protein in each preparation was determined, and then a portion of each was suspended in UDS-PMSF and, following solubilization at 37°C for 30 min, fractionated by 10% urea–SDS-PAGE and stained with silver. Estimates were made as to the fraction of the total soluble protein present as protoxin as described in Materials and Methods. Values for inclusions were 60% for *B. thuringiensis* subsp. *aizawai* HD133, 40% for strain 11, and 70% for *B. thuringiensis* subsp. *kurstaki* HD1 and strain CG3. For soluble protoxins, 70 to 80% was present as 130- to 135-kDa protein in all cases.

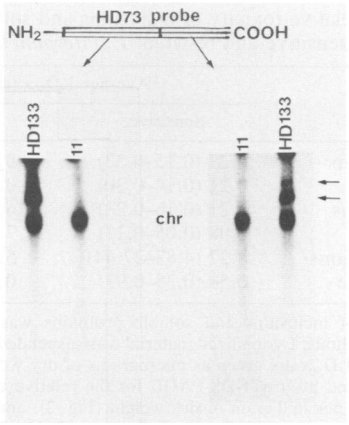

FIG. 1. Hybridization of DNA probes from the *cryIA(c)* gene to plasmids from *B. thuringiensis* subsp. *aizawai* HD133 and strain 11. Fragments from digests of the N_7 clone (see Materials and Methods) were used as probes. Arrows from top to bottom indicate hybridizing plasmids of ca. 150 and 68 kb. chr, Chromosomal DNA. Signal intensities for the same size plasmid vary because of differences in cell lysis and thus plasmid recovery.

dialyzed at 4°C against several changes of 0.03 M $NaHCO_3$ (pH 8.5). Inclusions were suspended in the latter buffer and sonicated very briefly to obtain uniform suspensions. For *P. interpunctella* bioassays, soluble protoxin was dialyzed extensively against deionized water before being lyophilized. The fraction of total protein in each extract or inclusion suspension (after solubilization in UDS-PMSF) present as 130- to 135-kDa protoxins was estimated by comparison with the staining intensities of various concentrations of bovine serum albumin electrophoresed on the same gel. These values are given in the footnote to Table 2.

Assays with first- or second-instar larvae of *T. ni*, *M. sexta*, or *H. virescens* on an artificial diet have been described elsewhere (4), and a similar diet was used for assays with *S. frugiperda* larvae. *P. interpunctella* sensitive and resistant to Dipel (25, 26) were assayed by a small-volume apple bioassay procedure (20). Calculations of 50% lethal doses (LD_{50}s) were done as previously described (4, 33).

Other procedures. Southern hybridizations were done with HCl-treated plasmids transferred from agarose gels (8) and with restriction enzyme digests. Probes were labeled by

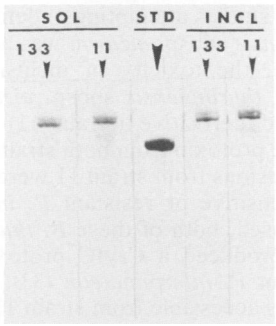

FIG. 2. Silver stain and urea-SDS-PAGE of proteins (used for the bioassays in Table 3) from soluble (SOL) or inclusion (INCL) preparations of *B. thuringiensis* subsp. *aizawai* HD133 and strain 11. Standards (STD) were β-galactosidose and bovine serum albumin (barely visible). Two hundred micrograms of each preparation was solubilized in UDS-PMSF, and 1/100 of the soluble extracts and 1/50 of the inclusion extracts were electrophoresed.

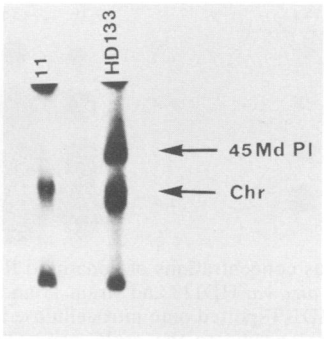

FIG. 3. Hybridization of a 584-bp *Eco*RI fragment from the variable region of the *cryIA(b)* gene from clone p121 to plasmids as for Fig. 1. Arrows indicate the 68-kb (45 MdP1) plasmid and chromosomal DNA (Chr). Hybridizing bands at the bottom of the strip are probably results of degraded DNA.

random primer extension (Amersham kit) or polynucleotide kinase (23). RNA was isolated as previously described, and dot blots were prepared with various concentrations (4). Hybridizations were to the ^{32}P-labeled probes previously described.

RESULTS

Protoxin gene composition. *B. thuringiensis* subsp. *aizawai* HD133 contained protoxin genes related to the *cryIA* class on at least two plasmids of ca. 68 and >150 kb (Fig. 1) and produced protoxins of two distinguishable sizes (Fig. 2). As discussed in Materials and Methods, a *cryIA(b)* gene was cloned from this subspecies and appears to be present on the 68-kb plasmid, since a 0.58-kb *Eco*RI fragment from the variable region of this cloned gene hybridized with the 68-kb but not the >150-kb plasmid from *B. thuringiensis* subsp. *aizawai* HD133 (Fig. 3). It is therefore likely that a CryIA(b)-type protoxin of ca. 131 kDa is one of the protoxins produced by *B. thuringiensis* subsp. *aizawai* HD133.

Strain 11, the plasmid-cured derivative of *B. thuringiensis* subsp. *aizawai* HD133, still contained a protoxin gene(s) on the >150-kb plasmid (Fig. 1) but lacked the plasmid-encoded *cryIA(b)* gene (Fig. 3), even though the *cryIA(b)*-specific probe hybridized with DNA from this strain. As a result of the curing, strain 11 no longer produced *cryIA(b)* mRNA (Fig. 4), so the DNA hybridizing with the *cryIA(b)* probe in

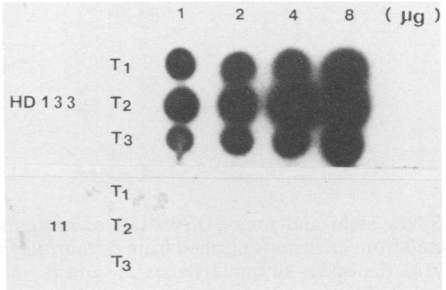

FIG. 4. Hybridization of the 584-bp *Eco*RI fragment from the *cryIA(b)* gene to various concentrations of denatured RNA from *B. thuringiensis* subsp. *aizawai* HD133 and strain 11 spotted onto nitrocellulose. T_1, Time when more than 60% of the cells contained phase white endospores; T_2 and T_3, 1.5 and 3 h later, respectively.

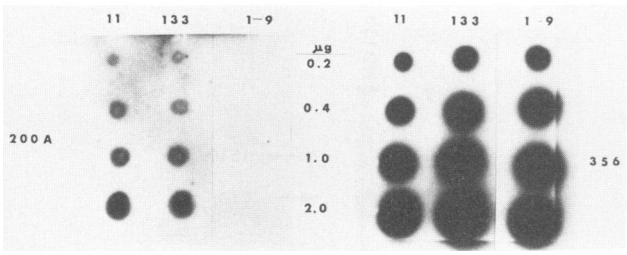

FIG. 5. Various concentrations of denatured RNA from *B. thuringiensis* subsp. *aizawai* HD133 and strain 11 and *B. thuringiensis* subsp. *kurstaki* HD1-9 spotted onto nitrocellulose and hybridized to an oligonucleotide specific to the *cryIC* gene (200A) and to one reacting with both *cryIA(b)* and *cryIC* mRNAs (356; see Materials and Methods). RNA was prepared from cells when about 80% contained phase white endospores.

the chromosomal fraction (Fig. 3) is likely to be due to a cryptic gene. There were still two size classes of protoxins produced by strain 11, although the relative intensities of the two bands appeared to be more nearly equal than those from *B. thuringiensis* subsp. *aizawai* HD133 (Fig. 2).

Both *cryIC* and *cryID* genes have been reported to be present in other *B. thuringiensis* subsp. *aizawai* strains (15). Since the CryIC toxin is effective on resistant *Plodia* spp. (33), it was likely that the *B. thuringiensis* subsp. *aizawai* strains studied here contained this gene, and so, as described in Materials and Methods, a *cryIC* gene was cloned from strain 11 plasmid DNA. In addition, an oligonucleotide specific for the *cryIC* gene (200A) hybridized to the >150-kb plasmid from *B. thuringiensis* subsp. *aizawai* HD133 and strain 11 (unpublished results), and mRNA hybridizing to this probe was produced by both strains in approximately equal amounts (Fig. 5). An oligonucleotide (356) hybridizing to both *cryIC* and *cryIA(b)* mRNA hybridized to RNA from both strains but more extensively to HD133 RNA, which is consistent with the absence of *cryIA(b)* mRNA from strain 11.

An oligonucleotide specific for the *cryID* gene (5′ ATAG CACTTTCAGCAGCAG) was prepared from a unique region of the sequence (14). This probe hybridized to denatured DNA and to RNA from *B. thuringiensis* subsp. *aizawai*

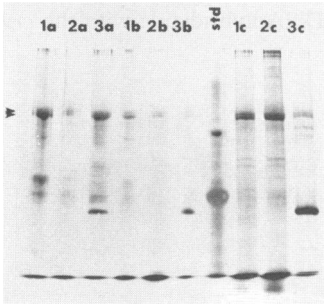

FIG. 6. Silver stain and urea-SDS-PAGE of proteins (5 µg of each) extracted from inclusions purified from *B. thuringiensis* subsp. *aizawai* HD133 (lanes 1), strain 11 (lanes 2), and *B. thuringiensis* subsp. *kurstaki* HD1 (lanes 3). Sequential extraction conditions (as in Table 4) were 0.03 M Na_2CO_3–0.1% βME (pH 9.2) (lanes a), 0.03 M Na_2CO_3–0.5% βME (pH 9.5) (lanes b) and UDS-PMSF (lanes c). Arrows indicate two bands of 130 to 135 kDa in all three preparations. Major bands of ca. 60 kDa in lanes 3a, 3b, and 3c are probably the CryIIA protoxin. Standards (std) are as in Fig. 2.

TABLE 3. Relative toxicity of inclusions and soluble protoxins for sensitive and resistant *P. interpunctella*

Source of protoxin	Average LD_{50}s (range)[a]	
	Sensitive	Resistant
HD133 inclusions	0.23 (0.15–0.32)	0.60 (0.44–0.82)
HD133 soluble	0.21 (0.14–0.30)	0.32 (0.23–0.43)
HD1-9 inclusions	0.21 (0.16–0.28)	16.09 (9.91–54.28)
HD1-9 soluble	0.08 (0.05–0.11)	7.48 (5.24–11.64)
Strain 11 inclusions	8.27 (4.83–37.44)	5.78 (4.11–9.62)
Strain 11 soluble	0.58 (0.35–0.93)	0.26 (0.15–0.41)

[a] Preparation of inclusions and soluble protoxins was as described in Materials and Methods. Lyophilized material was suspended for the bioassays (20, 33), and the LD_{50}s are given as micrograms of dry weight. All preparations were analyzed by urea-SDS-PAGE for the relative content of 130- to 135-kDa protoxin per milligram of dry weight (Fig. 2), and the values were comparable to those reported in the footnote to Table 2. Data from two separate experiments were combined.

HD133 and strain 11 but not to DNA from *B. thuringiensis* subsp. *kurstaki* HD1 (unpublished results). In addition, soluble protoxin from strain 11 was active on *M. sexta* but not *H. virescens* larvae, as expected for a strain lacking a CryIA(b) protoxin but containing the CryID protoxin (15; Table 2).

There were two size classes of protoxins from inclusion extracts of *B. thuringiensis* subsp. *aizawai* HD133 (Fig. 2 and 6), presumably the larger CryIC protoxin and a mixture of the CryIA(b) and CryID protoxins. Inclusion extracts from strain 11 also resolved into two size classes, but the staining intensities were more nearly equal, apparently because of the absence of the CryIA(b) protoxin. Overall, the data are consistent with the presence of CryIA(b), CryIC, and CryID protoxins in *B. thuringiensis* subsp. *aizawai* HD133 and only CryIC and CryID protoxins in strain 11. As previously mentioned, these *B. thuringiensis* subsp. *aizawai* strains also appeared to contain a cryptic *cryIA* gene perhaps similar to that in *B. thuringiensis* subsp. *entomocidus* 601 (31).

Toxicity of inclusions versus that of soluble protoxin. A protoxin produced by a *cryIA(b)* gene cloned from *B. thuringiensis* subsp. *aizawai* HD133 (p121; as described in Materials and Methods) was active on sensitive but not resistant *P. interpunctella*, as were inclusions from a *B. cereus* transcipient (strain 9) containing the *cryIA(b)* gene on a 68-kb plasmid from *B. thuringiensis* subsp. *aizawai* HD133 (12). In the initial studies attempting to define which toxin(s) from *B. thuringiensis* subsp. *aizawai* was active on resistant *P. interpunctella*, the toxicity of inclusions and soluble protoxin from *B. thuringiensis* subsp. *aizawai* HD133 and the plasmid-cured derivative (strain 11) was determined (Table 3). Soluble protoxin from both strains was active, but surprisingly, inclusions from strain 11 were much less effective for either sensitive or resistant *P. interpunctella*. As previously discussed, both of these *B. thuringiensis* subsp. *aizawai* strains produced a CryIC protoxin known to be active on resistant *P. interpunctella* (33), and so this protoxin must be less accessible from strain 11 inclusions. This pattern of difference between soluble protoxins and inclusions from strain 11 was also found in bioassays of *T. ni* and *M. sexta*, but toxicity was nearly equal in bioassays of *S. frugiperda* (Table 2). In all cases, inclusions and soluble protoxins from *B. thuringiensis* subsp. *aizawai* HD133 were about equally effective.

Solubility properties of inclusions differ. Purified inclusions

TABLE 4. Solubilization of inclusion protein

Buffer for sequential extraction	% of total protein solubilized[a]			
	B. thuringiensis subsp. *aizawai* HD133	Strain 11	*B. thuringiensis* subsp. *kurstaki* HD1	Strain CG3
0.03 M Na_2CO_3–0.1% βME (pH 9.2)	79	44	83	80
0.3 M Na_2CO_3–0.5% βME (pH 9.5)	8	11	3	5
UDS-PMSF[b]	12	45	14	15

[a] Average of two experiments. Extractions were done sequentially on the inclusion preparation, and protein was determined as described in Materials and Methods. There appeared to be no selective solubilization of a particular protoxin under any of the conditions used (Fig. 6).

[b] Total solubilization can also be achieved with 0.03 M Na_2CO_3–2% βME (pH 9.7), as for the bioassays of soluble protoxin (Tables 2 and 3).

were extracted sequentially with buffers, and the protein concentrations in the soluble fractions were determined (Table 4). A portion of each was also fractionated by urea-SDS-PAGE, and there was no evidence for selective solubilization of one of the molecular weight classes of 130- to 135-kDa protoxins from either strain with any of the solubilizing conditions employed (Fig. 6).

Solubility comparisons were also made with inclusions from *B. thuringiensis* subsp. *kurstaki* HD1 and one of its plasmid-cured derivatives, strain CG3. The latter lacks a 67-kb plasmid containing a *cryIA(b)* gene, so the bipyramidal inclusions should contain only CryIA(a) and CryIA(c) protoxins in contrast to the three protoxins in the parental strain (4, 22, 24). The solubility properties of these inclusion proteins were similar to those of proteins from *B. thuringiensis* subsp. *aizawai* HD133 (Table 4), and the inclusions and soluble protoxins were equally effective for *T. ni* (Table 2). In this case, the absence of a CryIA(b) protoxin (and the plasmid containing this gene) had no effect on inclusion protein solubility.

DISCUSSION

The specificity profile of most *B. thuringiensis* isolates is complex because of the presence of multiple protoxin genes and various proportions of two or more protoxins (4, 24). Related protoxins of 130 to 135 kDa are probably present in a single inclusion, whereas the smaller CryII protoxins of 60 to 65 kDa are present in distinct cuboidal inclusions (35). *B. thuringiensis* subsp. *aizawai* HD133 produced at least three protoxins [CryIA(b), CryIC, and CryID] as determined by hybridization of gene-specific probes to RNA from sporulating cells (Fig. 4 and 5) as well as SDS-PAGE of inclusion proteins (Fig. 2 and 6). Strain 11 lacked the 68-kb plasmid containing a functional *cryIA(b)* gene (Fig. 1 and 4). The RNA hybridization data, protoxins of two sizes, and the toxicity profile (Table 2) were all consistent with this strain containing CryIC and CryID protoxins. The presence of only two (strain 11) or three (*B. thuringiensis* subsp. *aizawai* HD133) protoxin genes in these strains was supported by the hybridization of an oligonucleotide probe (30-mer) prepared from a region encoding a highly conserved hydrophobic domain near the amino end of all protoxins sequenced to date (15, 34). This probe hybridized with only three *Hin*dIII fragments from total DNA from *B. thuringiensis* subsp. *aizawai* HD133 and with two from strain 11 (unpublished results). Given the extensive conservation of this region, the number of fragments hybridizing should reflect the total number of protoxin genes in each of these strains (unless *Hin*dIII fragments containing this region from various genes are of the same or a very similar size).

The presence of a cryptic *cryIA*-type gene in these *B. thuringiensis* subsp. *aizawai* strains was inferred from the hybridization of "chromosomal" DNA from strain 11 to a *cryIA(b)* probe (Fig. 3), the absence of *cryIA(b)* mRNA in this strain (Fig. 4), and the altered toxicity pattern as discussed above. Other strains of *B. thuringiensis* subsp. *aizawai* and at least one from *B. thuringiensis* subsp. *entomocidus* (31) contain a cryptic *cryIA* gene. In these cases, either an IS*231* sequence (8a) or other DNA had been inserted into the coding region. Results of Southern hybridization with DNA from *B. thuringiensis* subsp. *aizawai* HD133 and strain 11 were consistent with such an event, i.e., the presence of *Hin*dIII and *Nsi*I fragments of strain 11 DNA which hybridized with the *cryIA(b)* probe but were larger than expected from the known restriction enzyme sites in this gene (9, 22; unpublished results). The reason for the presence of cryptic protoxin genes is not known, but they could serve as a reservoir for genetic exchange.

The difference in protoxin composition and solubility properties between the two *B. thuringiensis* subsp. *aizawai* strains is probably due only to the curing of a 68-kd plasmid containing a *cryIA(b)* gene, since two other 42°C-cured derivatives of *B. thuringiensis* subsp. *aizawai* HD133 were isolated and both lacked the 68-kb plasmid and produced inclusions with solubility properties very similar to those of inclusions from strain 11. It is possible that factors encoded by this plasmid other than the CryIA(b) protoxin could influence protoxin solubility or the availability of a particular toxin (such as the susceptibility of inclusions per se to gut proteases and thus conversion to toxins). The in vitro solubility differences (Table 4), however, are consistent with the importance of this property to toxicity.

In the case of inclusions from *B. thuringiensis* subsp. *kurstaki* HD1 and the strain cured of a 67-kb plasmid containing a *cryIA(b)* gene (CG3), there was no change in the solubility of the CryIA protoxins in these inclusions. Another factor encoded by the 67-kb plasmid from *B. thuringiensis* subsp. *kurstaki* HD1, such as a nonprotoxin component of inclusions which could influence solubility, was either not required or not present. Inclusion solubility differences for *B. thuringiensis* subsp. *aizawai* strains, therefore, are more likely to be due to protoxin composition. These *B. thuringiensis* subsp. *aizawai* strains contain CryIC and CryID protoxins, which differ substantially in sequence (15) and perhaps in solubility from the CryIA types. If the latter is true, the CryIA(b) protoxin may function even in the absence of toxicity for resistant *Plodia* spp. to help solubilize the effective protoxin. Attempts are now being made to introduce a cloned *cryIA(b)* gene into strain 11 in order to establish the importance of this protoxin to inclusion solubility.

ACKNOWLEDGMENTS

Our research was supported by Public Health Service grant GM2606 from the National Institutes of Health and by a grant from the *B.t.* Management Working Group.

The assistance of Gail Sudlow, Sven Strnad, and Brian Barnett is appreciated.

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